UNIVERSITI PUTRA MALAYSIA

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERISATION OF AEROMONAS SPECIES FROM FISH

NOORLIS AHMAD

FSMB 2001 17

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERISATION OF *AEROMONAS* SPECIES FROM FISH

By

NOORLIS AHMAD

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Science Universiti Putra Malaysia

June 2001

SPECIALLY DEDICATED TO :

My beloved

Grandmother, abah, emak , adik,

relatives and friends

for your support .....

Thank You Very Much.....

Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERISATION OF *AEROMONAS* SPECIES FROM FISH

By

NOORLIS AHMAD

June 2001

Chairman : Associate Professor Son Radu, Ph. D.

Faculty : Food Science and Biotechnology

A total of 60 isolates of *Aeromonas* species which were originally isolated from various fish samples obtained from wet markets in Selangor were examined and further characterised by both phenotypic (antibiotics resistance and hemolysis assay) and genotypic (plasmid profiling, RAPD pattern and SDS-PAGE) methods. All the isolates examined in this study exhibited multiple antibiotic resistance pattern to antibiotics (ampicillin (98.4%), carbenicillin (93.6%), erythomycin (91.9%), bacitracin (87.1%), streptomycin (74.2%), kanamycin (58.1%), gentamycin (53.2%), tetracycline (46.8%), cephalothin (33.9%), nalidixic acid (25.8%), ceftriaxone (76.1%), cefoperazone (14.5%) and ceptazidime (8.06%)) tested. Plasmid analysis showed that 38.3%, 20%, 16.7% and 8.3% of isolates from Ikan Tilapia Merah, Ikan Keli, Ikan Terubuk and Ikan Merah respectively contained plasmid DNA bands with sizes ranging from 1.7 to 10.4 megadalton (MDa). Based on their plasmid profiles, the isolates of the *Aeromonas* species isolates were grouped into 18 plasmid patterns. Three 10-mer oligonucleotides primers GEN 1-50-02 (5'-CAATGCGTCT-3'), GEN1-50-06 (5'-CGGATAACTG-5')

and GEN1-50-08 (5'-GGAAGACAAC-3') were used to amplify genomic DNA. The profiles observed after electrophoretic separation for the 3 primers when combined together were able to distinguish the *Aeromonas* species isolates into 4 major clusters, respectively. In haemolysis assays of *Aeromonas* species,71.7% were observed to be alpha (α), 21.7% were beta (β) and only 6.7% were gamma (γ) haemolytic. The SDS-PAGE of whole cell protein pattern analysis indicated that the strains of *Aeromonas hydrophila* group have a dominant band of variable molecular weight between 25 to 67 kDa. Thus, the strains of *Aeromonas* species examined from various types of fish are genotypically diverse as shown by RAPD and SDS-PAGE protein pattern, suggesting that different strains have been brought into this geographical region or strains already present have continued to evolve. These results suggest that RAPD-PCR assay and SDS-PAGE whole cell protein pattern are more powerful methods than plasmid profiling and antibiotic resistance technique for discriminating *Aeromonas* species. Thus, RAPD-PCR assay and SDS-PAGE whole cell protein can be used as a valuable tool for epidemiological studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PEMENCILAN, IDENTIFIKASI DAN PENCIRIAN SECARA MOLEKUL SPESIS *AEROMONAS* DARIPADA IKAN

Oleh

NOORLIS AHMAD

Jun 2001

Pengerusi : Profesor Madya Son Radu, Ph.D.

Fakulti : Sains Makanan dan Bioteknologi

Sejumlah 60 pencilan spesis *Aeromonas* yang dipencilkan daripada pelbagai jenis ikan yang didapati daripada pasar-pasar di Selangor dikaji dan seterusnya dicirikan dengan kaedah "phenotypic" (antibiotik dan asai hemolisis) dan "genotypic" (profil plasmid, polimorfik menggunakan analisis RAPD dan SDS-PAGE). Semua pencilan yang digunakan di dalam kajian ini didapati memaparkan kepelbagaian corak terhadap kerintangan antibiotik (ampicillin (98.4%), carbenicillin (93.6%), erythomycin (91.9%), bacitracin (87.1%), streptomycin (74.2%), kanamycin (58.1%), gentamycin (53.2%), tetracycline (46.8%), cephalothin (33.9%), nalidixic acid (25.8%), ceftriaxone (76.1%), cefoperazone (14.5%) and ceptazidime (8.06%)) yang diuji. Profil plasmid yang diperolehi menunjukkan 38.3%, 20%, 16.7% dan 8.3% pencilan untuk Ikan Tilapia Merah, Ikan Keli, Ikan Terubuk dan Ikan Merah masing-masing mengandungi plasmid yang berada pada julat saiz antara 1.7 hingga 10.4 megadalton (MDa). Berdasarkan profil plasmid, pencilan spesis *Aeromonas* dapat dikumpulkan kepada 18 corak plasmid masing-masing. Tiga primer oligonukleotid 10-mer iaitu GEN 1-50-02 (5'-

CAATGCGTCT-3'), GEN1-50-06 (5'-CGGATAACTG-5') dan GEN1-50-08 (5'-GGAAGACAAC-3') digunakan untuk mengamplifikasikan genomik DNA. Penggabungan ketiga-tiga profil plasmid dapat membezakan kesemua spesis *Aeromonas* yang diuji kepada 4 kumpulan utama. Kajian hemolisis yang dijalankan ke atas semua pencilan *Aeromonas* di dalam kajian menunjukkan 71.7% pencilan hemolisis jenis alfa (α), sementara 21.7% jenis beta (β) dan hanya 6.7% jenis gama (γ) sahaja. Dengan menggunakan teknik analisis SDS-PAGE bagi profil protein sel, spesis *Aeromonas* mempunyai beberapa jalur dominan dengan berat molekul diantara 25 hingga 67 kDa. Pencilan spesis *Aeromonas* yang diperolehi dari pelbagai jenis ikan adalah berbeza seperti yang ditunjukkan oleh corak RAPD dan SDS-PAGE. Keputusan ini mencadangkan bahawa pencilan-pencilan yang sedia ada terus mengalami proses evolusi. Keputusan keseluruhan kajian ini menunjukkan bahawa teknik RAPD-PCR dan SDS-PAGE adalah lebih berkesan dari teknik profil plasmid dan kerintangan terhadap antibiotik untuk mendiskriminasikan spesis *Aeromonas*. Oleh itu RAPD-PCR dan SDS-PAGE boleh digunakan sebagai kaedah atau teknik yang amat berguna di dalam bidang kajian epidemiologi.

ACKNOWLEDGEMENTS

I would particularly like to express my deepest gratitude to my supervisor **Associate Professor Dr. Son Radu** for his continuous support, patience, invaluable advice, and guidance through out the course of this project. My deepest gratitude to my co-supervisors, **Dr. Foo Hooi Ling** and **Dr. Abdul Reezal Abdul Latif**. Without their persistent assistance and exceptional generosity, this work would not have been possible and meet the requirement of the course.

Special thanks are extended to all the postgraduate students of Foodborne Pathogen and Molecular Subtyping Group for their continuous support and assistance through out the whole project. Heart felt thanks to those helpful people of the Department of Biotechnology and Department of Food Science, and Graduate School (GSO) who have assisted and supported me either directly or indirectly through out the whole duration of my project and writing up for the final thesis.

Last but not least of all, a special note of thanks to my family (Abah, Emak and Adik), friends (Yanti, Wai Ling, Kqueen, Sam, Pak Herman, Zaza, Mr. Naseer, Ibu Endang, Kak Zila, Kak Zu, En. Zainuri and Kamil) and last but not least, all of my housemates (Ku Zah, Pura, Julia, Kak Ta, Yeen, Ina, P-Nat, Deeda, Lyna and Ja) who share much of my joy and sorrow. Words just cannot express my gratitude for their love, concern, support and patience that have sustained me throughout the whole course of my postgraduate studies.

I certify that an Examination Committee met on 19th June 2001 to conduct the final examination of Noorlis Ahmad on her Master of Science thesis entitle "Isolation, Identification and Molecular Characterisation of *Aeromonas* species Isolated from Fish" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of Examination Committee are as follows :

Suhaimi Napis, Ph.D
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Chairman)

Son Radu, Ph.D
Associate Professor,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

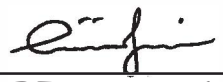
Foo Hooi Ling, Ph.D
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

Abdul Reezal Abdul Latif, Ph.D
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

MOHD GHAZALI MOHAYIDIN, Ph.D,
Professor
Deputy Dean of Graduate School,
Universiti Putra Malaysia.

Date : 22 JUN 2001

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.



AINI IDERIS, Ph.D,
Professor
Dean of Graduate School,
Universiti Putra Malaysia.

Date :

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

(NOORLIS AHMAD)

Date : 20 June 2001

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		v
ACKNOWLEDGEMENTS		vii
APPROVAL SHEETS		viii
DECLARATION FORM		x
LIST OF TABLES		xiv
LIST OF FIGURES		xv
LIST OF PLATES		xvii
LIST OF ABBREVIATIONS		xviii
CHAPTER		
1	INTRODUCTION	1
2	LITERATURE REVIEW	5
	2.1 Taxonomy	5
	2.2 Characteristics of Organisms	7
	2.3 Ecology	7
	2.4 Occurrence in Foods	8
	2.5 Survival and Growth	8
	2.5.1 Temperature	8
	2.5.2 Salt, pH and Additives	9
	2.5.3 Atmosphere	10
	2.6 Clinical Manifestations	11
	2.6.1 Extra Intestinal Infections	11
	2.6.2 Gastroenteritis	11
	2.7 Pathogenicity and Virulence Properties	12
	2.7.1 Enterotoxins	12
	2.7.2 Invasins	13
	2.7.3 Adhesins	14
3	ISOLATION AND IDENTIFICATION	16
	3.1 Introduction	16
	3.2 Materials and Methods	19
	3.2.1 Isolation	19
	3.2.2 Biochemical Tests	20
	3.3 Results	21
	3.4 Discussion	22
4	ANTIBIOTICS SUSCEPTIBILITY	24

4	ANTIBIOTICS SUSCEPTIBILITY		24
	4.1	Introduction	24
		4.1.1 Use of Antibiotic in Animals	24
		4.1.2 Current uses in Animal Agriculture	25
		4.1.3 Antibiotic Resistance	25
	4.2	Materials and Methods	27
	4.3	Results	28
	4.4	Discussion	32
5	PLASMID PROFILING		34
	5.1	Introduction	34
	5.2	Materials and Methods	37
	5.3	Results	38
	5.4	Discussion	41
6	HAEMOLYSIS		42
	6.1	Introduction	42
	6.2	Materials and Methods	43
	6.3	Results	43
	6.4	Discussion	46
7	RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) ANALYSIS		48
	7.1	Introduction	48
		7.1.1 Statistical Analysis	50
	7.2	Materials and Methods	52
		7.2.1 Genomic DNA Extraction	52
		7.2.2 RAPD-PCR Amplification	53
	7.3	Results	54
	7.4	Discussion	60
8	WHOLE CELL PROTEIN FINGERPRINTS		62
	8.1	Introduction	62
		8.1.1 Analysing the Protein Patterns Obtained	63
	8.2	Materials and Methods	64
		8.2.1 Whole Cell Protein Extraction	64
		8.2.2 SDS-PAGE Analysis	65
		8.2.3 Detection of Total Protein After Electrophoresis	66
		8.2.4 Drying the Gel	66
		8.2.5 Analysis of Electrophoresed Gel	67
	8.3	Results	67
	8.4	Discussion	68
9	GENERAL DISCUSSION		73
10	CONCLUSION		78

REFERENCES 80

VITAE 91

LIST OF TABLES

Table		Page
2.1	Current genospecies and phenospecies within the genus *Aeromonas*.	6
3.1	Differentiation among the *Aeromonas hydrophila* group.	18
3.2	Biochemical tests of *Aeromonas* species	20
3.3	Prevalence of *Aeromonas* species from various types of fish samples.	22
4.1	Antibiotic resistance of *Aeromonas* species isolated from fish.	29
4.2	Prevalence of resistance to antimicrobial agents amongst *Aeromonas* species isolated from various types of fish.	30
5.1	Plasmid profile of *Aeromonas* species isolates from fish.	40
6.1	Haemolysis of *Aeromonas* species isolated from various types of fish.	45
6.2	Types of haemolysis among *Aeromonas* isolates in relation to source of isolates.	45
7.1	Random primer screened in the random amplification polymorphic DNA (RAPD) procedure.	53

LIST OF FIGURES

Figure		Page
5.1	A representative photograph of the agarose (0.7%) gel electrophoresis of plasmid DNA from *A. veronii* biovar *sobria* isolated from fish. Lanes : M, V517 size reference plasmid ; lanes 1 to 9 : isolates of *A. veronii* biovar *sobria* numbered (ID) S1 to S9.	39
5.2	A representative photograph of the agarose (0.7%) gel electrophoresis of plasmid DNA from *A. veronii* biovar *sobria* and *A. caviae* isolated from fish. Lanes : M, V517 size reference plasmid ; lanes 1 to 8 : isolates of *A. veronii* biovar *sobria* and *A. caviae* numbered (ID) S43 to S48 and C1 to C2.	39
7.1	A representative of RAPD fingerprinting profile obtained using the GEN1-50-02 for the first 24 isolates of *A. veronii* biovar *sobria*. Lanes M : Molecular mass size marker of 1Kb DNA ladder; Lanes 1-24 : Isolates numbered (ID) S1 to S24.	56
7.2	A representative of RAPD fingerprinting profile obtained using the GEN1-50-02 for 10 isolates of *A. hydrophila* followed by 2 isolates of *A. caviae*. Lanes M: Molecular mass size marker of 1Kb DNA ladder; Lanes 1-10: Isolates numbered (ID) H1 to H10. Lanes 11: Molecular mass size marker of 1Kb DNA ladder; Lanes 12-13 : Isolates numbered (ID) C1 and C2.	56
7.3	A representative RAPD fingerprinting profile obtained using the GEN1-50-06 for the first 24 isolates of *A. veronii* biovar *sobria*. Lanes M: Molecular mass size marker of 1Kb DNA ladder; Lanes1-24 : Isolates numbered (ID) S1 to S24.	57
7.4	A representative RAPD fingerprinting profile obtained using the GEN1-50-06 for 10 isolates of *A. hydrophila* followed by 2 isolates of *A. caviae*. Lanes M and 11 : Molecular mass size marker of 1Kb DNA ladder; Lanes 1-10 : Isolates numbered (ID) H1 to H10. Lanes 12-13 : Isolates numbered (ID) C1 and C2.	57

7.5 A representative RAPD fingerprinting profile obtained using the GEN1-50-08 for the last 24 isolates of *A. veronii* biovar *sobria*. Lanes M: Molecular mass size marker of 1Kb DNA ladder; Lanes 1-24 : Isolates numbered (ID) S25 to S48. 58

7.6 A representative RAPD fingerprinting profile obtained using the GEN1-50-08 for 10 isolates of *A. hydrophila* followed by 2 isolates of *A. caviae*. Lanes M: Molecular mass size marker of 1Kb DNA ladder; Lanes 1-10 : Isolates numbered (ID) H1 to H10. Lanes 12-13 : Isolates numbered (ID) C1 and C2. 58

7.7 The dendrogram generated from matrix data obtained from RAPD profile . The patristic distance showed the genetic dissimilarity among the isolates of *Aeromonas* species used in this study. ... 59

8.1 A representative SDS-PAGE fingerprinting of whole cell protein for *A. veronii* biovar *sobria*. Lanes M: Broad range molecular weight markers (2-212 kDa). Lanes 1-7 : Isolates numbered (ID) S1 to S7 71

8.2 A representative SDS-PAGE fingerprinting of whole cell protein for *A. hydrophila*. Lanes M: Broad range molecular weight markers (2-212 kDa). Lanes 1-6 : Isolates numbered (ID) H1 to H6. 71

8.3 Dendrogram shows the clustering of SDS-protein electrophoregrams of 60 isolates of *Aeromonas* species using the UPGMA method. 72

LIST OF PLATES

Plate		Page
4.1	Disc-diffusion method to determine the antimicrobials susceptibility test (Bauer *et al.*, 1966). Each disc contains a different antibiotics, which diffuses into the surrounding agar. The clear zone indicate inhibition of growth of the *Aeromonas* species swabbed onto the plate surface.	31

LIST OF ABBREVIATIONS

%	percentage
β	beta
α	alpha
γ	gamma
μg	microgram
μl	microlitre
Am	ampicillin
AMP	adenosine 3', 5'- monophosphate
AP-PCR	arbitrarily primed- polymerase chain reaction
APS	ammonium persulphate
APW	alkaline peptone water
B	bacitracin
BAA	blood ampicillin agar
BHIA	brain heart infusion agar
BSA	Bovine serum albimun
C	chloramphenicol
^{0}C	degree Celsius
Caz	ceptazidime
Cb	carbenicillin
Cf	ceptrialothin

Cfu	colony forming unit
Cfp	cefoperazone
CO_2	carbon dioxide
Cro	ceftriaxone
DNA	deoxyribonucleic acid
dNTP	deoxynucleic triphosphate
E	erythromycin
E. coli	*Escherichia coli*
e.g.	for example
EDTA	ethylenediamine tetraacetic acid
EPEC	enteropathogenic *Escherichia coli*
EtBr	ethidium bromide
ETEC	enterotoxigenic *Escherichia coli*
F	fertility
g	gram
G+C	guanine + cytosine
GET	glucose-EDTA-tris buffer
Gm	gentamicin
GSP	glutamate starch phenol-red agar
H_2	hydrogen
HCl	Hydrochloric acid
HGs	hybridisation groups

K	kanamycin
Kb	kilobase
kDa	kilodalton
LB	Luria Bertani
LT	Heat-labile toxin
M	molar
MDa	megadalton
Mg	miligram
$MgCl_2$	magnesium chloride
ml	mililitre
Mol	mole
Na	nalidixic acid
NaCl	sodium chloride
ND	non detected
ng	nanogram
no.	number
Nor	norfloxacin
PBS	phosphate buffer saline
PCI	phenol chloroform isoamylalcohol
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
BIBG	bile-salts-irgasan-brilliant green agar
POR	plasmid occurrence rate

pmol	picomole
R	resistant
RNA	ribonucleic acid
rpm	revolution per minute
S	streptomycin
SAA	starch ampicillin agar
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sp.	species
Taq	*Thermus aquaticus* DNA (polymerase)
TBE	tris-boric-acid-EDTA
Te	tetracycline
TEMED	N,N,N,N, tetramethylethylene-diamine
Tris	tris (hydroxymethyl methylamine)
TSBA	typticase soy broth agar
UV	ultra violet
V	volts
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Foodborne disease has become a topic of much recent attention as reported incidence of gastrointestinal disease worldwide has increased dramatically during the 1990s. Various organisms such as *E. coli*, *Shigella*, *Vibrio* and *Aeromonas* have been isolated. The genus *Aeromonas* was proposed first by Kluyver and Van Niel in 1936 (Popoff, 1984). The genera *Aeromonas*, *Vibrio*, *Photobacterium* and *Pleisomonas* are included in the family *Vibrionaceae*. On the basis of molecular genetic evidence, proposals have been made to divide the genus *Aeromonas* in a new family, *Aeromonadaceae* (Kuijper *et al.*, 1989).

The genus *Aeromonas* consists of two groups of organisms ; (1) a single nonmotile species (*Aeromonas salmonicida*) that is pathogenic to fish but not human, and (2) several motile species (the *Aeromonas hydrophila* group) that are associated with human illness. Based on biochemical characteristics and DNA relatedness, *Aeromonas hydrophila* group has been divided into 3 species ; *Aeromonas hydrophila*, *Aeromonas sobria* and *Aeromonas caviae*. Bacteria of the *A. hydrophila* group occur widely in aquatic environments, belong to the flora of reptiles, amphibian and fish, and have been implicated in the aetiology of a variety of systematic and localised diseases in fish and reptiles (Burke *et al.*, 1984; Palumbo and Buchanan, 1988; Palumbo *et al.*, 1989; Kirov *et al.*, 1990; Ibrahim and Mac Rae, 1991; Walker and Brooks, 1993; Son *et al.*, 1997).

A. hydrophila group has received particular attention because of its association with soft tissue and disseminated infectious and acute or chronic gastroenteritis following ingestion of contaminated food or water (Son *et al.*, 1997). This group of organism is also pathogenic to many aquatic species and causes hemorrhagic septicaemia (red sore disease) in many fresh water pond-cultured and wild native fish (Abeyta *et al.*, 1986). Other spectrum of infections by *Aeromonas* species including otitis, eye infections, tonsilitis, pneumonia, urinary tract infections, osteomyelitis and meningitis. This broad spectrum of infections is paralleled by a range of virulence factors including adhesins, cytotoxins, hemolysis, and various enzymes (Donna and Lindsey, 1988).

Drug resistant in *Aeromonas* species is well known. Animals reared in aquaculture facilities are susceptible to numerous bacterial diseases, which can be treated with a variety of antimicrobial compounds. The extensive use of antibiotics and other chemotherapeutics in fish farms as feed additives or the direct administration thereof into fishpond water to prevent and treat fish diseases, has resulted in an increase of drug-resistant bacteria as well as R plasmids. Increased incidence of bacterial resistance to standard antibiotic treatments has been recognised, particularly in fish shipped from Asia (Son *et al.*, 1997). More over, there remains the possibility that resistance may be transmitted from antibiotic-resistant bacteria to the susceptible ones (Imziln *et al.*, 1996).

For the identification of the sources and monitoring the spread of *Aeromonas* species, a number of epidemiology markers, including various molecular characterisation techniques such as antibiotype, plasmid profile, polymerase chain reaction, pulsed field gel electrophoresis (PFGE), protein profile, phage typing and classical electrophoresis of DNA-restricted digests are useful to determine the genetic relatedness among the determined isolates under study. Nowadays, polymerase chain reaction (PCR) is the most common technique used to study the characteristics of bacteria. The PCR reaction shows differences in-between species or strains by analysing the size of the DNA products amplified from genomic DNA templates by a variety of primers. In higher organism, sets of random primers have been used to generate random amplified polymorphic DNA (RAPD)-PCR products, which produce banding patterns, when separated on agarose gels, that are characteristics of in-between species or isolates (Smith *et al.*, 1998).

In this study, *A. hydrophila*, *A. veronii* biovar *sobria* and *A. caviae* isolated from fish are used as the *Aeromonas* species of interest.